

Biochemical Effects of Hexabromocyclododecane (HBCD) diastereoisomers in Juvenile Rainbow trout (*Onch orhynchus mykiss*)

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Introduction

Brominated flame retardants are widely used and increasingly detected in abiotic and biotic compartments globally. Tetrabromobisphenol, polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) are most ubiquitous. HBCD is the principle fire retardant in polystyrene foams that are used as insulation in the building industry and for upholstering furniture. Bioaccumulation of HBCD in animals and humans has caused concern regarding the potential for health effects (Zegers et al. 2006, Tomy et al. 2004). Among these potential health effects, disturbance of the thyroid axis has recently been reported (Yamada-Okabe et al. 2005).

The molecular structure of HBCD is shown in Figure 1. The technical mixture consists of three diastereoisomers, α , β and γ . The γ isomer is most often detected, but there can be substantial differences in the composition of HBCD residues that are measured in biota.

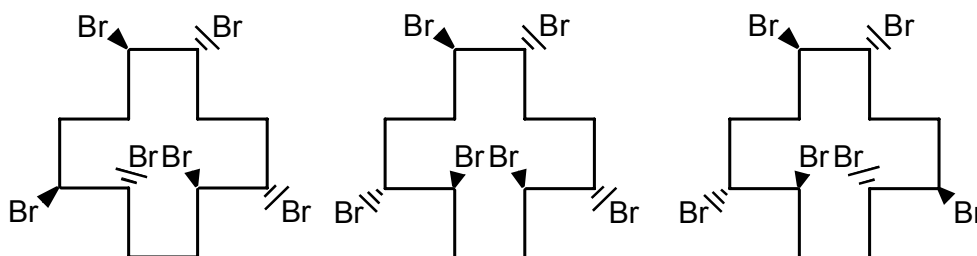


Figure 1: Structures of the α -, RR,SR,RS (left), β -, RR,SR,SR (middle) and γ -, RR,RS,SR (right) HBCD isomers.

Tomy et al. (2004) recently reported differences in the proportions of HBCD isomers in a Great Lakes food web, but there is little information on the relative potency of the diastereoisomers to induce biological effects in exposed organisms. To address this deficiency, juvenile rainbow trout (*Oncorhynchus mykiss*) were held in the laboratory and fed diets containing environmentally relevant concentrations of the individual α , β and γ isomers. At different stages of the exposure, fish were sacrificed and tissues were harvested to assess bioaccumulation (reported in Law et al. 2006) and possible biochemical effects of HBCD.

Materials and Methods.

Food preparation. The preparation of the fish food has been described previously (Tomy et al. 2003). Four batches of food were prepared in this study: three of the batches were spiked with a known amount of a particular diastereoisomer while none of the diastereoisomers were added to the reference diet. Lipid corrected concentrations of α , β , γ -isomer in the food were determined to be 29.14 ± 1.95 , 11.84 ± 4.62 and 22.84 ± 2.26 ng/g, respectively. Respective lipid corrected concentrations of α - and γ -HBCD in the control food were 0.47 and 0.84 ng/g; the β -isomer was

below method detection limits. Food was stored in the dark at -4°C to exclude the possibility of phototransformations.

Fish. Juvenile rainbow trout with initial mean weights of 233 ± 89 g were randomly separated into four 800 L fibreglass aquaria receiving water at a constant water flow of 1.5 L/min of UV- and carbon-dechlorinated Winnipeg City tap water, at a temperature of 11-12°C and pH between 7.9 and 9.1. The dissolved oxygen was always at level of saturation. A 12-h light 12-h dark photoperiod was maintained throughout the experiment. Fish were acclimatized for 7 days prior to the start of the experiment. Fish in each of the three aquaria were exposed to an individual HBCD isomer *via* their food; fish in the fourth tank were exposed to control food (i.e., unfortified and having undetectable concentrations of any of the diastereoisomers) throughout the experiment. There was an uptake phase of 56 days, where fish were fed fortified food, followed by a depuration phase of 112 days, where all fish were fed unfortified food. The feeding rate was 1% of their body weight three times a week. This rate was adjusted after each sampling day based on the mean weight of the fish sacrificed. Four fish from each tank were sacrificed on days 0, 7, 14, and 56 of the uptake phase and days 7, 14, 56, and 112 of the depuration phase. Fish were sacrificed 48 hours after the previous feeding by an overdose of a pH buffered solution of MS-222 (0.4 g/L). Once operculum movement ceased, 3-5 mL of blood was removed *via* the caudal vein, along with the liver, kidney, muscle tissue and thyroid. Only tissue from the carcass was used for calculation for bioaccumulation parameters. Details of the extraction and analysis of muscle tissue can be found in Law *et al.* (2006). Liver was used for screening for phase I cytochrome P450 and deiodinase enzyme activities.

Biochemical and Histological Analysis. Preparation of microsomes from liver tissue and analysis of Phase I enzyme activity, as ethoxyresorufin-O-deethylase (EROD), in samples from Day 0, 14 and 56 of the uptake phase and days 14 and 56 of the depuration phase were performed as previously described by Palace *et al.* (1998). Briefly, approximately 0.5 g of liver tissue was added to 10 volumes of ice cold 0.1 M Tris-HCl buffer (pH=7.6) that contained 0.1M NaCl. Livers were homogenized for 30 seconds with a Polytron homogenizer model PT 10/35 (Brinkman Instruments Inc., Westbury, NY). The homogenate was centrifuged at 10,000 g for 20 minutes and the resulting supernatant was recovered and re-centrifuged at 105,000 g for 90 minutes. The pellet was recovered by pouring off the supernatant and the pellet was resuspended in 1 ml of 0.05 M Tris Buffer (pH=7.6) and frozen in microcentrifuge tubes at -90°C until analysis. EROD activity and protein content in the microsomes were determined simultaneously using the method of Kennedy and Trudeau (1994). Fluorescence detection in this system was linear for at least 8 minutes. Free triiodothyronine (T3) and thyroxine (T4) were determined on days 0, 7, 14, 28 and 56 of the uptake phase and day 112 of the depuration phase using 100 µL of plasma in a standard coated tube radioimmunoassay (ICN Pharmaceuticals, Orangeburg, NY). T4 outer ring deiodinase enzyme activity (T4ORD) was determined in liver microsomes as described by Eales *et al.* (1999). Blanks were included in each analytical run to account for non-specific release of ¹²⁵I from T4. Thyroid gland histology (epithelial cell height) was evaluated as described in Brown *et al.* (2004).

Data Analysis. Details regarding the diastereoisomer concentration calculations can be found in Law *et al.* (2006). Growth rates, assimilation efficiencies, depuration rates, half lives and BMFs are as published in Tomy *et al.* (2004). Group means were compared for EROD and T4ORD were compared by ANOVA followed by Dunnett's test. Statistical significance was accepted at $p < 0.05$.

Results and Discussion.

No mortalities occurred throughout this experiment in fish from any of the experimental treatments. Growth rates of the control and exposed fish ranged between 0.11 and 0.16 g d⁻¹ and there were also no statistically significant differences between the groups. Liver size was not significantly different among fish from any of the groups at any of the sample times (Figure 2). Enlarged livers have been reported in juvenile rainbow trout exposed to HBCD for 28 days, but only at exposures that were much higher (ie. at ~ 500 but not at 50 mg/kg bodyweight) (Ronisz et al. 2004) than the doses employed in this study (~10-30 µg/ kg bodyweight).

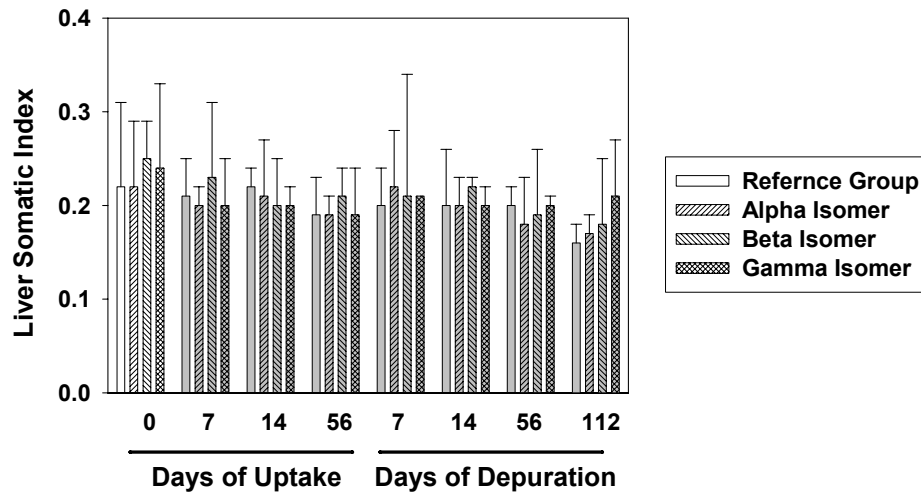


Figure 2: Liver somatic index ($=(\text{liver wt (g)}/\text{carcass wt (g)}) \times 100$) during uptake and depuration phases in fish fed the reference food as well as fish fed the food enriched with alpha, beta or gamma HBCD isomer. Data are expressed as Mean \pm SEM (n=4, p>0.05).

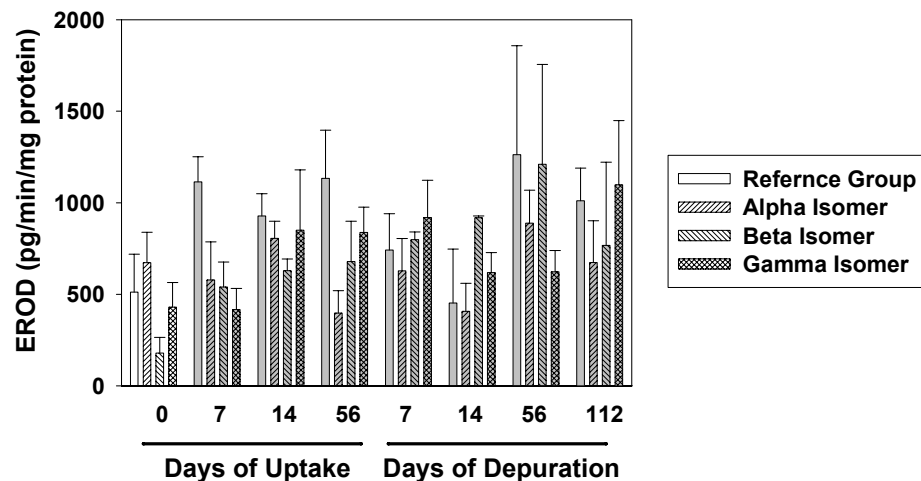


Figure 3: Ethoxyresorufin-O-deethylase (EROD) activity in liver of juvenile rainbow trout fed a reference diet or diets containing alpha, beta or gamma HBCD isomers. Data are expressed as Mean \pm SEM (n=4, p>0.05) * = significantly different than other treatments for the same sample period.

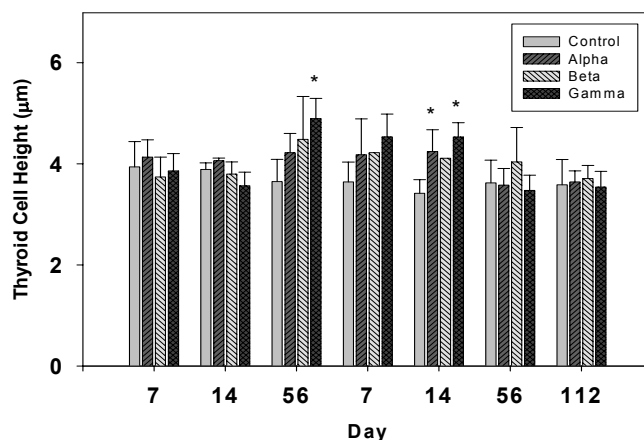


Figure 4: Thyroid epithelial cell heights in juvenile rainbow trout fed a reference diet or diets containing alpha, beta or gamma HBCD isomers. Data are expressed as Mean \pm Standard Deviation (n=4, p>0.05) * = significantly different than other treatments for the same sample period.

The reference group had significantly higher EROD activity compared to the HBCD exposed groups on days 7 and 56 of the uptake phase, but there was no consistent induction/inhibition of EROD in any HBCD group (Figure 3). Again, previous studies demonstrating induced EROD activity (Ronisz et al. 2004) have detected the responses only at much higher doses than those in the current study.

Thyroid epithelial cell heights were significantly greater in the γ -HBCD exposed group at day 56 of the uptake phase and in fish from the α - and γ -HBCD exposed group at day 14 of the depuration phase, relative to fish in the reference group (Figure 4). Enlarged thyroid epithelial cells are a measure of thyroid gland hypertrophy (Brown et al. 2004). A total examination of thyroid axis disruption includes measures of circulating thyroid hormone profiles as well as deiodinase enzyme activity. Analysis of these endpoints are pending and will be presented.

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